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Mini Review

Mini-Review: Important Roles of Chromatography in the Quantitation of Biomarkers Using Liquid Chromatography and Mass Spectrometry (LC-MS)

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Abstract

Due to the endogenous nature of biomarkers and many components similar to the target biomarkers present in the biological fluids, the analysis of biomarkers presents a series of added chromatographic challenges to bioanalytical scientists, ranging from poor sensitivity to inadequate selectivity. Optimization and application of an appropriate chromatographic condition played a critical role in those issue driven resolutions. In this mini-review paper, the author highlighted some of the practical solutions to overcome these chromatographic challenges using the relevant examples from author's laboratory. Future direction on further enhancing chromatographic selectivity for biomarker analysis, a pivotal bioanalytical parameter of endogenous biomarkers which is unique to biomarker analysis (in contrast to typical bioanalysis of drugs and metabolites which are exogenous compounds), will also be discussed.

Keywords: Endogenous Biomarker; Bioanalysis; Chromatography; LC-MS

Introduction

Endogenous compounds such as small molecules and peptide/proteins are being increasingly utilized as biomarkers to facilitate drug discovery and development processes [1]. Biomarkers are also used in environmental and human health risk assessment such as environmental tobacco smoke exposure [2,3]. Appropriate application of biomarker data can enable not only the predictive and reliable decision-making but also patient stratifications and regulatory filing. LC-MS techniques, due to their intrinsic selectivity and sensitivity, have been an important bioanalytical platform for biomarker quantization [4].

The principle of MS is the production of ions from analyzed compounds that are separated or filtered on the basis of their mass-to charge ratio (m/z). Most of applications for quantitative bioanalysis use tandem Mass Spectrometers (MS/MS) that employs two mass analyzers – one for the precursor ion in the first quadruple and the other for the product ion in the third quadruple after the collision – activated dissociation of the precursor ion in a collision cell. Innovative and successful research efforts in the past decades on the design of an effective interface connection between LC (operated under atmospheric pressure) and MS (operated under a high-vacuum environment) have made LC congenial with MS. Electro Spray Ionization (ESI) and Atmospheric-Pressure Chemical Ionization (APCI), collectively called Atmospheric Pressure Ionization (API), have matured into reliable interface necessary for routine quantitative LC-MS/MS bioanalysis.

Yet, analysis of analytes including drugs, metabolites and biomarkers in biological matrices using robust LC-MS/MS methods remains a difficult, time-consuming, daunting, and occasionally dismaying challenge. Successful use of LC-MS/MS requires understanding the mechanism of various sample extraction processes and the underlying principles of both chromatography and MS [5].

Typically prior to chromatography, biological samples are subjected to what we usually called "sample extraction" using Solid-Phase Extraction (SPE), Liquid-Liquid Extraction (LLE), or Protein Precipitation (PPT). The purpose of the sample extraction is to reduce interferences which can co-elute with the analytes and cause quantitation errors or sensitivity loss either via interference or matrix effect. Typically a more thorough clean-up and use of automation in this stage would lead to cleaner samples for the injection and shorter chromatographic run time [6]. Nevertheless, due to the inherent nature of less efficient separation power, complete remove interferences during sample extraction is infeasible. Besides, harsh, excessive and tedious steps in the sample extraction process may also lead to other bioanalytical pitfalls including but not limited to analytes loss due to degradation, evaporation or non-specific adsorption to the container; conversion of conjugated biomarkers to biomarkers which may lead to artificial over-estimation of the biomarker. Therefore, utilization of the superior resolution power of the chromatography still plays the critical role in developing a robust bioanalytical LC-MS method for the endogenous biomarker quantization.

Bioanalysis LC-MS of endogenous biomarkers

Due to its endogenous nature and many similar components including possible isomers of the target biomarkers present in the biological fluids, the analysis of biomarkers presents a series of added chromatographic challenges to bioanalytical scientists, ranging from poor sensitivity to inadequate selectivity. In this mini-review paper, the author highlighted some of the practical solutions to overcome some of these chromatographic challenges using the relevant examples from author's laboratory. It is only fair to mention that many other scientists also contribute significantly to the field of bioanalysis of biomarkers using LC-MS but their publications are not included here

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due to the nature of an editorial paper. Selection of an appropriate chromatographic condition played a critical role in those issue driven resolutions. Future direction on further enhancing chromatographic selectivity for biomarker analysis, a pivotal bioanalytical parameter of endogenous biomarkers, will also be discussed.

The primary goal of chromatography in bioanalysis of biomarkers is to separate the analytes of interest from interference peaks such as other endogenous interferences, and any other components such as phospholipids since these compounds can negatively impact the quantitation of the analytes.

While some of these compounds can be visibly viewed on the chromatogram (for example, isobaric interference of the biomarkers or in-source breakdown of Phase II metabolites such as glucuronide conjugates), most of them are invisible at the detection channel (ion suppression from phospholipids and co-eluting non-isobaric endogenous interferences). In an ideal world, biomarkers should be chromatographically well resolved from all other compounds except the internal standard which is only in the form of heavy labeled biomarker of interest. Approaches for chromatographic resolution can be achieved by having better column efficiency (higher plate count or lower plate height) and/or better chromatographic resolution (higher peak capacity).

Chromatographic efficiency and selectivity

In order to understand the chromatographic separation, it is important to review the basic chromatography theory on Van Demeter equation.

$$H = A + B/u + Cu$$

A term represents the contribution from eddy diffusion. Eddy diffusion results from radial flow inequalities through a packed bed.

B term (B/u) represents the contribution from longitudinal diffusion.

C term (Cu) represents the contributions from resistance to mass transfer in the stationary and mobile phases.

u is mobile phase velocity (mm/s).

An optimum mobile phase velocity exists for a column at which its highest efficiency would be realized. While the A term is somewhat fixed for each column, the B term and C term played significant role in the column efficiency. For any given column, due to the longitudinal diffusion, tremendous efficiency loss will be observed when operating under a very low velocity, something to be aware of when using nano-flow chromatography. C term is more related to the characteristics of the analytes. While low mobile phase velocity is more favorable for C term, it was counter-balanced by the unfavorable B term. At higher velocity, while the impact from longitudinal diffusion is minimized, the mass transfer in and out of the stationary phase can plays a more significant role. In particular, analytes with high molecular weights such as protein and peptides biomarkers can have quite unfavorable mass transfer at high velocity. For these large analytes, lower mobile phase velocity, larger pore sizes (300 Å or larger instead of typical 80-100 Å for small molecules), smaller particle sizes, and elevated column temperature (to accelerate mass transfer) are often employed. Larger pore size and smaller particles have shorter diffusion path lengths, allowing a salute to travel in and out of the particle faster. Therefore the analytes spends less time inside the particle where peak diffusion can occur. Of course, column with smaller particles, especially those sub-1.7 μm, will need a pumping system that can handle high column back pressure.

Peak capacity is another very useful measurement for the overall chromatography selectivity and efficiency. Peak capacity is defined as the maximum number of peaks that can be theoretically separated on a column at given chromatographic conditions. Selectivity and column efficiency need to be optimized to maximize peak capacity in the one-dimensional separation. The highest peak capacity is obtained if all peaks are evenly spaced and column efficiency remains unchanged. Selectivity optimization can be achieved by changing mobile phase composition, changing column temperature or changing composition of stationary phase. This approach represented the classic chromatography optimization and is still a preferred way to chromatographically multiple isobaric interferences for the biomarker quantitation. Sufficient selectivity does not guarantee good efficiency as two well-separated peaks may have very broad peak shape. Therefore, a complementary approach is to optimize column efficiency, which can be usually realized by using smaller particle column and minimizing system extra volume. While this approach is an easier and faster way to separate analytes from each other or from matrix components, one may still need to be aware that going to smaller particles may not improve the selectivity and therefore the space between the analytes. The improved separation is solely from reduced plate count which may be compromised upon repeated injections of biological extracts onto the column. It was reported that going from a 3.5 μm column to a 1.8 μm column only improved peak capacity by 80% with a caveat of 5- fold increase on column back pressure [7]. To achieve optimal chromatographic separation, both good selectivity and high efficiency are required.

Utilization of chromatographic principles for biomarker assay selectivity

A good example of achieving desirable selectivity is illustrated by the separation of 4-β hydroxyl-cholesterol from 4-α hydroxyl-cholesterol as the former is being investigated as a potential biomarker for CYP450 3A4/5 inducers. The later can be formed upon auto-oxidation during sample processing [8,9]. Another excellent example is the analysis of Leukotriene B_4 (LTB_4) in human plasma. LTB_4 is an important inflammatory component in a number of diseases and has been used as a Pharmacodynamic (PD) biomarker. Its isobaric endogenous isomers include 6-epi LTB_4, 12-trans LTB_4 and 6-epi-12-trans LTB_4. Chromatographic separation of LTB_4 from these isobaric isomers is essential, otherwise LTB_4 concentration will be severely over-estimated since all these compounds share the identical transition in tandem mass spectrometer. The chromatographic separation of LTB_4 from its isomers is mainly achieved using the selectivity feature provided by different brands of the reversed-phase C18 column [10,11]. An earlier work from our laboratory also indicated that one may take the advantage of the selectivity difference between different brands of reversed-phase column to maximize the selectivity [12]. S-Phenylmercapturic acid is widely accepted as a specific biomarker for the evaluation of benzene exposure. Its chromatographic resolution from the interference peaks from human urine largely depends on the reversed-phase column selection.

Approaches for enhancing sensitivity on biomarker analysis

Another unique challenge of LC-MS/MS analysis of biomarkers is the poor sensitivity because of lack of good ionization of some biomarkers due to either lack of a precharged function group in the molecule or lack of chromatographic retention on a typical reversed-phased column due to its extremely polar nature. In the former situation, derivatization to add a char-gable function group is often used as a tool to enhance sensitivity. This was successfully employed in our laboratory for a number of neutral biomarkers including 4-β hydroxyl-cholesterol and 1-Hydroxypyrene (1-OHP). 1-OHP is a biomarker for pyrene (a smoking biomarker) and is present in the urine at pg/mL range. Due to lack of favorable MS fragmentation, 1-OHP itself can only be measured at ng/mL range without derivatization. A well-established derivatization strategy was used to enhance the method sensitivity. Dansyl chloride was used to enhance the sensitivity by introducing a moiety with favorable ionization and fragmentation. Low pg/ml sensitivity was achieved even on a Sciex API 3000 [13]. Poor chromatographic retention under a highly aqueous mobile phase lead to both the poor ionization efficiency and poor separation of interference which can cause ionization suppression and poor selectivity. In our laboratory, we have routinely successfully applied non-reversed-phase chromatographic stationary phase such as hydrophilic chromatographic chromatography to resolve this issue not just for polar biomarkers but also for many polar drugs and metabolites [14,15]. One particular interesting example is the analysis of nitrosamine. The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been suspected as a potential lung carcinogen in tobacco smoke. In vivo, NNK is rapidly metabolized to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL is a biomarker for exposure to NNK in tobacco smoke. Due to the polar nature of NNAL and poor retention, it is highly challenging to achieve 5 pg/ml sensitivity by using a traditional reversed-phase LC-MS/MS. A Betasil silica 50 x 3 mm, I.D., 5 μm column was used. Mobile phase consisted of acetonitrile, water, and formic acid, containing 5 mM ammonium acetate (95:5:0.1, v/v/v) at a flow rate of 1.0 ml/min. Low pg/ml sensitivity and excellent resolution of NNAL from other endogenous compounds were achieved with chromatographic run time only at 1 minutes [16]. Under Hydrophilic Interaction Chromatography (HILIC) condition, water is a stronger elution solvent than organic solvents. Some SPE elution solvents and LLE extracts can be injected directly onto LC-MS/MS [17]. Sensitivity is significantly improved because of increased organic content in the mobile phase.

High flow rate HILIC LC-MS analysis for biomarkers

High flow rate HILIC is also possible [18]. One example of combining the advantages of superior selectivity, high sensitivity, and fast analysis is demonstrated in the case of nicotinic acid (also known as vitamin B3 or niacin) analysis. Nicotinic Acid (NA) can be metabolized to its six metabolites, including Nicotinamide (NAM), Nicotine Uric Acid (NUA), 1-methyl-2-Pyridone-5-carboxamide (2-PY), 1-methyl-4-Pyridone-5-carboxamide (4-PY), 1-Methyl-Nicotinamide (1-MNAM), and Nicotin amide-N-Oxide (NAMO). Nicotinamide may be a biomarker for larval survival and development. Due to the similarity of the chemical structures for many of these analytes, chromatographic separation was important to avoid potential interference. An ultra-fast method for analysis these seven analytes in human plasma and urine was reported [19]. Initially, a high flow LC-MS/MS method on a RP monolithic C18 column was attempted, but the analytes were poorly retained. Even with a mobile phase containing only 5% (v: v) of acetonitrile, all the analytes eluted near the void volume. This was undesirable as the poorly retained analytes may encounter ion suppression from matrix components as well as isotopic/isobaric interference from each other. In comparison, HILIC demonstrated powerful separation capability for these polar molecules. Seven analytes were well retained on the column, and 2-PY and 4-PY were completely separated within 60 seconds using gradient elution at a flow rate of 4.0 mL/min. The maximum back-pressure during the gradient run was only 140 bars. Ultra-fast analysis can also significantly reduce column re-equilibrium time, which was only 0.2 min in this study. Excellent reproducibility for the gradient elution was achieved on the silica column. At least 1100 injections, including extracted plasma and urine samples prepared using PPT or SPE, were made onto the same column without noticeable change in retention time and peak shape.

Approach to ensure assay specificity

It is worthwhile to mention that in an LC-MS assay, endogenous analytes are more susceptible to specificity issues due to inference from isobaric compounds or structural analogs which may be generated from the same biological pathway as the targeted analytes. Based on knowledge of the targeted analytes, the matrix, and the biological pathway, different strategies can be attempted to confirm the specificity of an LC-MS assay [20,21]. A different retention mechanism, such as HILIC that is complimentary to reversed-phase LC, may be employed for better elucidation of components that were un-resolved under the intended chromatographic conditions. Ultra-Performance Liquid Chromatography (UPLC) with enhanced efficiency and resolution can also be used to confirm assay specificity [10]. A high-resolution mass spectrometry such as QTOF can also provide additional selectivity by using the much superior mass resolution power [22,23]. A newly introduced Differential Mobility Device (DMS, SelexION™ from Sciex) might be very useful for the added separation dimension [24]. It performs a gas phase differential ion mobility separation within the planar ion mobility cell based on the size and shape of the compound prior to entering the mass analyzer. It is attached between the curtain plate and orifice plate. DMS alternates between high field and low field mobility. A compensation voltage is applied at a filtering voltage which is specific for each ion being targeted. The separation power can be enhanced with the use of integrated organic modifiers such as Isopropanol, acetonitrile, and methanol. Its cycle times are compatible with multiple component analysis and fast analysis times. DMS can be used to increase selectivity and eliminates the background noise.

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